Supplementary Materials for

Olefin Cyclopropanation via Carbene Transfer Catalyzed by Engineered Cytochrome P450 Enzymes

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Olefin cyclopropanation via carbene insertion catalyzed by engineered cytochrome P450 enzymes

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I. Materials and Methods

Unless otherwise noted, all chemicals and reagents for chemical reactions were obtained from commercial suppliers (Sigma-Aldrich, Acros) and used without further purification. The following heme proteins were all purchased from Sigma-Aldrich: myoglobin (from equine heart), peroxidase II (from horseradish), cytochrome c (from bovine heart), catalase (from *Corynebacterium glutamicum*) and chloroperoxidase (from *Caldariomyces fumago*). Silica gel chromatography purifications were carried out using AMD Silica Gel 60, 230-400 mesh. ^{1}H and ^{13}C NMR spectra were recorded on either a Varian Mercury 300 spectrometer (300 MHz and 75 MHz, respectively), or a Varian Inova 500 MHz (500 MHz and 125 MHz, respectively), and are internally referenced to residual solvent peak. Data for ^{1}H NMR are reported in the conventional form: chemical shift (δ ppm), multiplicity (s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, br=broad), coupling constant (Hz), integration. Data for ^{13}C are reported in terms of chemical shift (δ ppm) and multiplicity. High-resolution mass spectra were obtained with a JEOL JMS-600H High Resolution Mass Spectrometer at the California Institute of Technology Mass Spectral Facility. Reactions were monitored using thin layer chromatography (Merck 60 silica gel plates) using an UV-lamp for visualization. Optical rotation was measured using a JASCO P-2000 Polarimeter.

Gas chromatography (GC) analyses were carried out using a Shimadzu GC-17A gas chromatograph, a FID detector, and J&W scientific cyclosil-B columns (30 m x 0.32 mm, 0.25 μm film and 30 m x 0.25 mm, 0.25 μm film). High-performance liquid chromatography (HPLC) was carried out using an Agilent 1200 series, an UV detector, and an Agilent XDB-C18 column (4.6 x 150 mm, 5 μm). Cyclopropane product standards for the reaction of ethyl diazoacetate (EDA) with styrene (ethyl 2-phenylcyclopropane-1-carboxylate) and α-methylstyrene (ethyl 2-methyl-2-phenylcyclopropane-1-carboxylate) were prepared as reported (*18*). These standards and enzyme-prepared cyclopropanes demonstrated identical retention times in gas chromatograms when co-injected, confirming product identity. Absolute stereoconfiguration of cyclopropane enantiomers was determined by measuring optical rotation of purified cyclopropane products from preparative bioconversion reactions using enantioselective P450-BM3 variants and referenced to values taken from reference (*19*). Authentic P450-catalyzed cyclopropane samples were also prepared as described in section VIII and were characterized by NMR (^{1}H and ^{13}C) and mass spectrometry.

Plasmids pCWori[BM3] and pET22 were used as cloning vectors. Site-directed mutagenesis was accomplished by standard overlap mutagenesis using primers bearing desired mutations (IDT, San Diego, CA). Primer sequences are available upon request. Electrocompetent *Escherichia coli* cells were prepared following the protocol of Sambrook *et al.* (*20*). Restriction enzymes BamHI, EcoRI, XhoI, Phusion polymerase, and T4 ligase were purchased from New England Biolabs (NEB, Ipswich, MA). Alkaline phosphatase was obtained from Roche (Nutley, NJ). The 1,000x trace metal mix used in expression cultures contained: 50 mM $FeCl_3$, 20 mM $CaCl_2$, 10 mM $MnSO_4$, 10 mM $ZnSO_4$, 2 mM $CoSO_4$, 2 mM $CuCl_2$, 2 mM $NiCl_2$, 2 mM Na_2MoO_4, and 2 mM H_3BO_3.

II. General Procedures

Enzyme library screening. Libraries maintained in our laboratory are stored at -78 °C as glycerol stocks (Luria-Bertani medium (LB_{amp}), 150 µL, 25% v/v glycerol with 0.1 mg mL^{-1} ampicillin) in 96-well plates. These stocks were used to inoculate 96-shallow-well plates containing 300 µL LB_{amp} medium using a 96-pin stamp. Single colonies from site-saturation libraries were picked with toothpicks and used to inoculate 300 µL of LB_{amp}. The cells were incubated at 37 °C, 250 rpm shaking, and 80% relative humidity overnight. After 16 h, 50 µL aliquots of these overnight cultures were transferred into 2 mL, deep-well plates containing terrific broth (TB_{amp}) (800 µL containing 0.1 mg mL^{-1} ampicillin, 1 µL mL^{-1} trace metal mix and 20 mg L^{-1} aminolevulinic acid) using a Multimek 96-channel pipetting robot (Beckman Coulter, Fullerton, CA). The cultures were incubated at 37 °C for 3 h and 30 min, and 30 min after reducing the incubation temperature to 25 °C (250 rpm, 80% relative humidity), 50 µL isopropyl β-D-1-thiogalactopyranoside (IPTG, 4.5 mM in TB_{amp}) was added, and the cultures were allowed to continue for another 24 h at 25 °C (250 rpm, 80% relative humidity). Cells were then pelleted (3,000xg, 15 min, 4 °C) and stored at -20 °C until further use, but at least for 2 h. For cell lysis, plates were allowed to thaw for 30 min at room temperature and then cell pellets were resuspended in 275 µL phosphate buffer (0.1 M, pH = 8.0, 0.65 mg mL^{-1} lysozyme, 10 mM magnesium chloride and 40 U mL^{-1} DNAse I). The lysing cells were incubated at 37 °C for 1 h. Cell debris was separated by centrifugation at 5,000xg and 4 °C for 15 min. The resulting crude lysates were then transferred to 96-well microtiter plates for CO assays and to 2 mL deep-well plates for bioconversions.

CO binding assay. $Na_2S_2O_4$ (160 µL, 0.1 M in phosphate buffer, 0.1 M, pH = 8.0) was added to $P450_{BM3}$ variants in cell lysate (40 µL). The absorbance at 450 and 490 nm was recorded using a Tecan M1000 UV/Vis plate reader, and the microtiter plates were placed in a vacuum chamber. The chamber was sealed, evacuated to approximately - 15 in Hg, purged with CO gas, and incubated for 30 min. The plates were then removed and the absorbance at 450 and 490 nm was again recorded using a plate reader. The difference spectra could then be used to determine the P450 concentration in each well as previously described (*21*).

P450 expression and purification. For the enzymatic transformations, $P450_{BM3}$ variants were used in purified form. Enzyme batches were prepared as follows. One liter TB_{amp} was inoculated with an overnight culture (100 mL, LB_{amp}) of recombinant *E. coli* DH5α cells harboring a pCWori plasmid encoding the P450 variant under the control of the *tac* promoter. After 3.5 h of incubation at 37 °C and 250 rpm shaking (OD_{600} ca. 1.8), the incubation temperature was reduced to 25 °C (30 min), and the cultures were induced by adding IPTG to a final concentration of 0.5 mM. The cultures were allowed to continue for another 24 hours at this temperature. After harvesting the cells by centrifugation (4 °C, 15 min, 3,000xg), the cell pellet was stored at -20 °C until further use but at least for 2 h. The cell pellet was resuspended in 25 mM Tris.HCl buffer (pH 7.5 at 25 °C) and cells were lysed by sonication (2x1 min, output control 5, 50% duty cycle; Sonicator, Heat Systems - Ultrasonic, Inc.). Cell debris was removed by centrifugation for 20 min at 4 °C and 27,000xg and the supernatant was subjected to anion exchange chromatography

on a Q Sepharose column (HiTrapTM Q HP, GE Healthcare, Piscataway, NJ) using an AKTAxpress purifier FPLC system (GE healthcare). The P450 was eluted from the Q column by running a gradient from 0 to 0.5 M NaCl over 10 column volumes (P450 elutes at 0.35 M NaCl). The P450 fractions were collected and concentrated using a 30 kDa molecular weight cut-off centrifugal filter and buffer-exchanged with 0.1 M phosphate buffer (pH = 8.0). The purified protein was flash-frozen on dry ice and stored at -20 °C. P450 concentration was determined in triplicate using the CO binding assay described above (10 μL P450 and 190 μL 0.1 M phosphate buffer, pH 8.0, per well).

Thermostability measurements. Duplicate measurements were taken for all values reported on Tables S10 and S11. Purified P450 solutions (4 μM, 200 μL) were heated in a thermocycler (Eppendorf) over a range of temperatures (38 °C - 65 °C) for 10 min followed by rapid cooling to 4 °C for 1 min. The precipitate was removed by centrifugation. The concentration of folded P450 remaining in the supernatant was measured by CO-difference spectroscopy (as described above). The temperature at which half of the protein was denatured (T_{50}) was determined by fitting the data to the equation: $f(T) = 100 / (1 + \exp(a*(T - T_{50})))$.

Typical procedure for small-scale cyclopropanation bioconversions under anaerobic conditions. Small-scale reactions (400 μL) were conducted in 2 mL crimp vials (Agilent Technologies, San Diego, CA). P450 solution (80 μL, 100 μM) was added to the vial with a small stir bar before crimp sealing with a silicone septum. Phosphate buffer (260 μL, 0.1 M, pH = 8.0) and 40 μL of a solution of the reductant (100 mM $Na_2S_2O_4$, or 20 mM NADPH) were combined in a larger crimp-sealed vial and degassed by bubbling argon through the solution for at least 5 min (Fig S1). In the meantime, the headspace of the 2 mL reaction vial with the P450 solution was made anaerobic by flushing argon over the protein solution (with no bubbling). When multiple reactions were conducted in parallel, up to 8 reaction vials were degassed in series via cannulae. The buffer/reductant solution (300 μL) was syringed into the reaction vial, while under argon. The gas lines were disconnected from the reaction vial before placing the vials on a plate stirrer. A 40x styrene solution in MeOH (10 μL, typically 1.2 M) was added to the reaction vial via a glass syringe, and left to stir for about 30 s. A 40x EDA solution in MeOH was then added (10 μL, typically 400 mM) and the reaction was left stirring for the appropriate time. The final concentrations of the reagents were typically: 30 mM styrene, 10 mM EDA, 10 mM $Na_2S_2O_4$, 20 μM P450.

The reaction was quenched by adding 30 μL HCl (3M) via syringe to the sealed reaction vial. The vials were opened and 20 μL internal standard (20 mM 2-phenylethanol in MeOH) was added followed by 1 mL ethyl acetate. This mixture was transferred to a 1.8 mL eppendorf tube which was vortexed and centrifuged (16,000xg, 1 min). The top organic layer was dried over an anhydrous sodium sulfate plug and analyzed by chiral phase GC.

A slightly modified work-up was implemented for kinetic experiments. The reactions were quenched after the set time by syringing 1 mL EtOAc to the closed vials and immediately vortexing the mixture. The vials were then opened and 20 μL internal standard was added. The mixture was transferred to a 1.8 mL eppendorf tube, vortexed

and centrifuged (16,000xg, 1 min). The top organic layer was dried over an anhydrous sodium sulfate plug and analyzed by GC.

Typical procedure for preparative-scale cyclopropanation bioconversions under anaerobic conditions. The P450 solution was added to a Schlenk flask with a stir bar. With the flask kept on ice, the head-space was evacuated and back-filled with argon (4 x) with care not to foam the protein solution. Phosphate buffer and reductant were pre-mixed and degassed together in a separate round-bottom-flask by bubbling argon through the solution for 20 min. The buffer/reductant solution was transferred to the Schlenk flask via syringe. Styrene was added under argon and left to mix for 1 min. EDA was added dropwise under argon. The solution was left to stir under argon until reaction completion. The reaction was quenched under argon by adding hydrochloric acid (3 M) to adjust the pH to 4, before opening the Schlenk flask. The reaction mixture was stirred with sodium chloride and dichloromethane (CH_2Cl_2). The combined emulsion layers were then filtered through Celite to break the emulsion and the Celite pad was rinsed with 3x20 mL CH_2Cl_2. The resulting biphasic mixture was transferred to a separating funnel and the organic phase was removed. The remaining aqueous phase was re-extracted with 3x40 mL CH_2Cl_2. The combined organic extracts were dried with sodium sulfate, filtered, and concentrated. The resulting residue was purified by SiO_2 chromatography.

III. Preliminary Experiments with Heme Proteins

The following six heme proteins were initially screened for cyclopropanation activity: catalase, chloroperoxidase (CPO), horseradish peroxidase (HRP), cytochrome C (cyt *c*), myoglobin (Mb) and $P450_{BM3}$. Small-scale (400 µL) reactions were conducted as described in section II and were analyzed by GC (cyclosil-B 30 m x 0.25 mm x 0.25 µm): oven temperature = 130 °C.

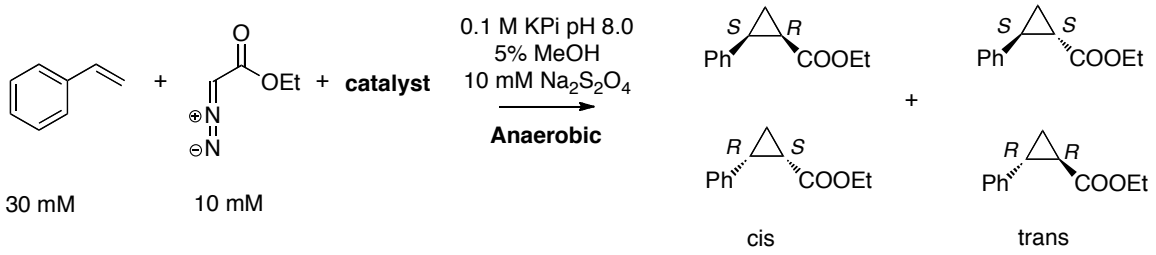


Table S1: Heme catalysts under **anaerobic** conditions **with sodium dithionite** ($Na_2S_2O_4$)

catalyst	axial ligand	cat. loading (% mol eq)	TTN	cis : trans*	%ee cis†	%ee trans‡
catalase	O-Tyr	0.16	0	-	-	-
CPO§	S-Cys	0.40	0	-	-	-
HRP	N-His	1.00	9	7 : 93	8	-7
cyt *c*	N-His, S-Met	1.00	19	6 : 94	0	12
Mb	N-His	1.00	43	6 : 94	-1	2
$P450_{BM3}$	S-Cys	0.20	5	37 : 63	-27	-2
hemin	-	0.20	73	6 : 94	-1	0

* Diastereomeric ratios and enantiomeric excess were determined by GC analysis. † (*R*,*S*) – (*S*,*R*). ‡ (*R*,*R*) – (*S*,*S*). § Bioconversion conducted at 0.1 M citrate buffer pH = 4.0.

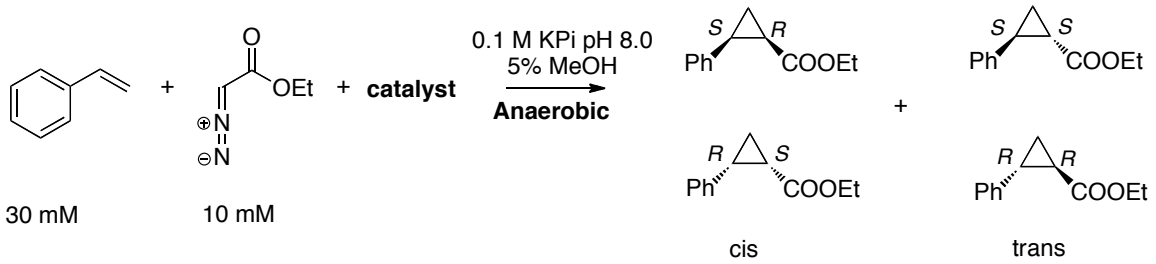


Table S2: Heme catalysts under **anaerobic** conditions **without $Na_2S_2O_4$**

catalyst	axial ligand	cat. loading (% mol eq)	TTN	cis : trans*	%ee cis†	%ee trans‡
catalase	O-Tyr	0.16	0	-	-	-
CPO§	S-Cys	0.40	0	-	-	-
HRP	N-His	1.00	0	-	-	-
cyt *c*	N-His, S-Met	1.00	12	8 : 92	8	-3
Mb	N-His	1.00	0.8	11 : 89	-2	8
$P450_{BM3}$	S-Cys	0.20	0	0	-	-
hemin	-	0.20	4	11 : 89	-1	3

∗ Diastereomeric ratios and enantiomeric excess were determined by GC analysis. † (*R*,*S*) – (*S*,*R*). ‡ (*R*,*R*) – (*S*,*S*). § Bioconversion conducted at 0.1 M citrate buffer pH = 4.0.

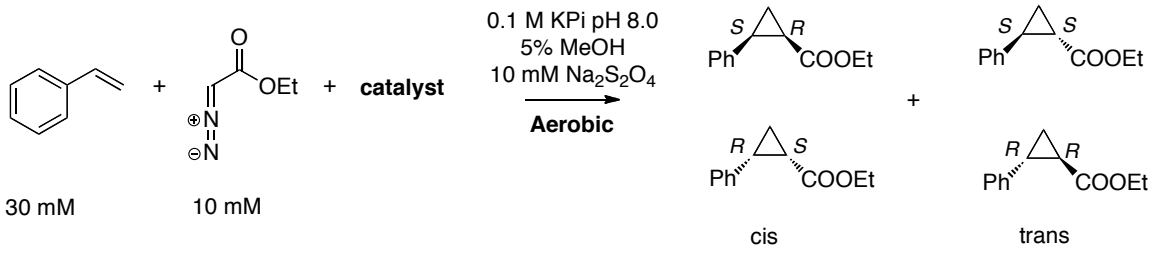


Table S3: Heme catalysts under **aerobic** conditions **with $Na_2S_2O_4$**

catalyst	axial ligand	cat. loading (% mol eq)	TTN	cis : trans*	%ee cis†	%ee trans‡
catalase	O-Tyr	0.16	0	-	-	-
CPO§	S-Cys	0.40	0	-	-	-
HRP	N-His	1.00	1	12 : 88	-3	-7
cyt *c*	N-His, S-Met	1.00	3	9 : 91	-6	16
Mb	N-His	1.00	6	7 : 93	-13	12
$P450_{BM3}$	S-Cys	0.20	1	13 : 87	-38	-8
hemin	-	0.20	6	8 : 92	-5	1

∗ Diastereomeric ratios and enantiomeric excess were determined by GC analysis. † (*R*,*S*) – (*S*,*R*). ‡ (*R*,*R*) – (*S*,*S*). § Bioconversion conducted at 0.1 M citrate buffer pH = 4.0.

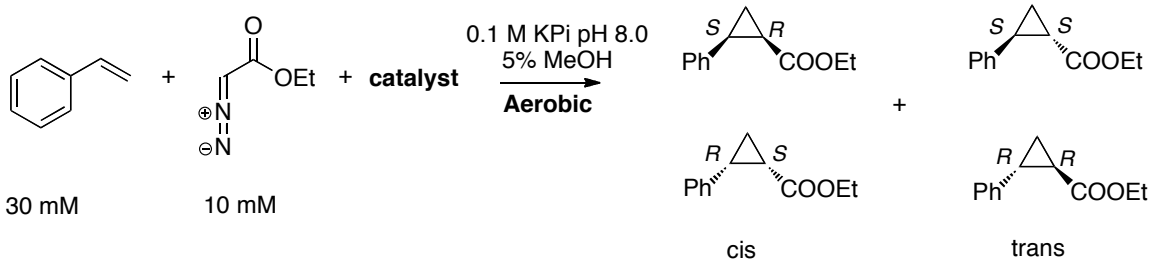


Table S4: Heme catalysts under **aerobic** conditions **without $Na_2S_2O_4$**

catalyst	axial ligand	cat. loading (% mol eq)	TTN	cis : trans*	%ee cis†	%ee trans‡
catalase	O-Tyr	0.16	0	-	-	-
CPO§	S-Cys	0.40	0	-	-	-
HRP	N-His	1.00	0	-	-	-
cyt *c*	N-His, S-Met	1.00	0	-	-	-
Mb	N-His	1.00	0	-	-	-
$P450_{BM3}$	S-Cys	0.20	0.4	46 : 54	-46	36
hemin	-	0.20	0	-	-	-

∗ Diastereomeric ratios and enantiomeric excess were determined by GC analysis. † (*R*,*S*) – (*S*,*R*). ‡ (*R*,*R*) – (*S*,*S*). § Bioconversion conducted at 0.1 M citrate buffer pH = 4.0.

IV. Screening $P450_{BM3}$ variants for cyclopropanation activity

Lysate screening under aerobic conditions. The 92 variants in our compilation plate (Table S5, following pages) represent a diverse selection of $P450_{BM3}$ variants that have previously been engineered for monooxygenase activity on a variety of substrates, including but not limited to short alkane hydroxylation, demethylation of protected monosaccharides, and oxidation of lead drug compounds. These $P450_{BM3}$ variants carry various mutations accumulated along sequential rounds of engineering efforts for activity towards the target substrates (Table S5) or were generated by recombination with homologous enzymes (Table S6). The compilation plate was expressed and lysed as described in section II (**enzyme library screening**). 150 µL lysate was transferred (Multimek 96-channel pipetting robot, Beckman Coulter, Fullerton, CA) to a 2 mL deep-well plate, with 50 µL of 120 mM $Na_2S_2O_4$ in 0.1 M KPi (pH = 8.0). 100 µL of a 30 mM styrene, 60 mM EDA mixed solution in 15% MeOH in 0.1 M KPi (pH = 8.0) was added to the plate to initiate the reaction. The plate was sealed and was left shaking (300 rpm) for four hours. The plastic seal was removed and 30 µL HCl (3 M) was added to quench the reaction followed by 20 µL of an internal standard solution (20 mM α-methylstyrene in methanol). The reactions were extracted by adding 500 µL EtOAc and carefully vortexing the plate. The plate was centrifuged (1,700xg) to separate the biphasic mixture. The top organic layer was transferred (2 x 150 µL) to a separate deep-well plate. The extracts for each of the 92 reactions were dried through 92 separate anhydrous sodium sulfate plugs. The dried extracts were analyzed by GC (cyclosil-B 30 m x 0.32 mm x 0.25 µm): oven temperature = 60 °C 3 min, 7.5 °C / min to 160 °C, 20 °C / min to 250 °C, 250 °C 2 min, *cis*-cyclopropanes (20.3 min and 20.45 min), *trans*-cyclopropanes (21.8 min). The top 10 protein variants of importance with respect to this report are highlighted in tables S5 and S6.

Table S5: Raw data from $P450_{BM3}$ compilation plate screen

Diastereo- and enantioselectivity were determined by gas chromatography using a chiral β-CDX column as the stationary phase.

$P450_{BM3}$ variant	**mutations with respect to wild-type $P450_{BM3}$**	**absolute activity***	***de*†**	***ee* (cis) ‡**
CYP102A3	N/A	0.004053	-74	-8
CYP102A2	N/A	0.002963	-76	-36
CYP102A1 ($P450_{BM3}$)	None	0.002240	-81	7
WT F87A	F87A	0.001704	-28	57
WT T88L	T88L	0.004522	-78	23
WT A328V	A328V	0.000830	-100	N/A
J (*22*)	V78A, T175I, A184V, F205C, S226R, H236Q, E252G, R255S, A290V, L353V	0.001334	-100	N/A
139-3 (*23*)	V78A, H138Y, T175I, V178I, A184V, F205C, S226R, H236Q, E252G, R255S, A290V, L353V	0.001386	-86	0
9-10A (*22*)	R47C, V78A, K94I, P142S, T175I, A184V, F205C, S226R, H236Q, E252G, R255S, A290V, L353V	0.004292	-74	-20
9-10A L75W (*24*)	**9-10A** L75W	0.005191	-83	-8
9-10A L75I (*24*)	**9-10A** L75I	0.002267	-85	-3
9-10A A78F (*24*)	**9-10A** L78F	0.002008	-82	-35
9-10A A78S (*24*)	**9-10A** A78S	0.005098	-81	-6
9-10A A82G (*24*)	**9-10A** A82G	0.002245	-76	-7
9-10A A82F (*24*)	**9-10A** A82F	#VALUE!	N/A	N/A
9-10A A82C (*24*)	**9-10A** A82C	0.002487	-74	16
9-10A A82I (*24*)	**9-10A** A82I	0.001031	-100	N/A
9-10A A82S (*24*)	**9-10A** A82S	0.001483	-82	14
9-10A A82L (*22*)	**9-10A** A82L	0.000591	-100	N/A
9-10A F87A	**9-10A** F87A	0.001701	-61	-10
9-10A F87V (*24*)	**9-10A** F87V	0.000000	N/A	N/A
9-10A F87I (*24*)	**9-10A** F87I	0.000983	-100	N/A
9-10A F87L (*24*)	**9-10A** F87L	0.000710	-100	N/A
9-10A T88C (*24*)	**9-10A** T88C	0.002516	-77	3
9-10A T260S (*24*)	**9-10A** T260S	0.004259	-82	-6
9-10A T260N (*24*)	**9-10A** T260N	0.003882	-77	15
9-10A T260L (*24*)	**9-10A** T260L	0.006173	-77	-2
9-10A A328V (*22*)	**9-10A** A328V	0.006471	-68	-8
9-10A A328M (*24*)	**9-10A** A328M	0.005180	-82	6
9-10A A328F (*24*)	**9-10A** A328F	0.002009	-63	-32

49-1A	R47C, V78T, A82G, F87V, K94I, P142S, T175I, A184V, F205C, S226R, H236Q, E252G, R255S, A290V, A328L, L353V	0.001874	-75	-32
35-7F	R47C, V78F, A82S, K94I, P142S, T175I, A184V, F205C, S226R, H236Q, E252G, R255S, A290V, A328L, L353V	0.004514	-73	-52
53-5H (*24*)	**9-10A** A78F, A82S, A328F	0.002840	-80	2
7-11D	**9-10A** A82F, A328V	0.036840	-24	-28
49-9B	R47C, V78A, A82G, F87V, K94I, P142S, T175I, A184V, F205C, S226R, H236Q, E252G, R255S, A290V, A328L, L353V	0.000000	N/A	N/A
41-5B	R47C, V78F, A82G, K94I, P142S, T175I, A184V, F205C, S226R, H236Q, E252G, R255S, A290V, A328V, L353V	0.008391	-77	-17
13-7C (*24*)	**9-10A** A78T, A328L	0.005493	-73	-43
12-10C	R47C, V78A, A82G, F87V, K94I, P142S, T175I, A184V, F205C, S226R, H236Q, E252G, R255S, A290V, A328V, L353V	0.004566	-73	-21
77-9H (*24*)	**9-10A** A78T, A82G, A328L	0.003053	-73	-34
11-8E	R47C, V78A, F87V, K94I, P142S, T175I, A184V, F205C, S226R, H236Q, E252G, R255S, A290V, A328L, L353V	0.001453	-77	15
1-12G (*22*)	**9-10A** A82L, A328V	0.003884	-70	-19
29-3E	R47C, V78A, A82F, K94I, P142S, T175I, A184V, F205C, S226R, H236Q, E252G, R255S, A290V, A328F, L353V	0.003425	-80	15
29-10E	R47C, V78F, A82G, K94I, P142S, T175I, A184V, F205C, S226R, H236Q, E252G, R255S, A290V, A328F, L353V	0.001935	-70	16
68-8F (*24*)	**9-10A** A78F, A82G, A328L	0.004127	-72	-32
35E11 (*25*)	R47C, V78F, A82S, K94I, P142S, T175I, A184V, F205C, S226R, H236Q, E252G, R255S, A290V, A328F, L353V, E464G, I710T	0.003600	-71	-14
19A12 (*25*)	**35E11** L52I, L188P, I366V	0.006909	-70	-27
ETS8 (*25*)	**35E11** L52I, I366V	0.003966	-79	-19
(11-3) (*25*)	**35E11** L52I, A74S, L188P, I366V	0.005633	-76	-39

(7-7) (*25*)	**35E11** L52I, A74E, S82G, A184V, L188P, I366V	0.010499	-77	-9
H2A10	**9-10A TS** F87V, L75A, L181A, T268A	0.066422	-8	-94
SL2-6F8	R47C, L52I, V78F, A82S, K94I, P142S,T175I, A184V, F205C, S226R, H236Q, E252G, R255S, A290V, A328L, K349N, L353V, I366V, E464G, I710T	0.000778	-100	N/A
A12SL-17-4	R47C, L52I, A74E, V78F, A82S, K94I, P142S,T175I, A184V, L188P, F205C, S226R, H236Q, E252G, R255S, A290V, A328F, L353V, I366V, E464G, I710T	0.010935	-80	6
H2-2-A1 (*26*)	**9-10A TS** F87V, L75A, L181A, L437A	0.003042	-75	-11
A12RM-2-8	R47C, L52I, A74E, V78F, A82S, K94I, P142S,T175I, A184S, L188P, F205C, S226R, H236Q, E252G, R255S, A290V, A328F, L353V, I366V, E464G, I710T	0.007705	-77	-13
H2-5-F10	**9-10A TS** F87V, L75A, I263A, T268A, L437A	0.141237	-46	-56
13C9R1	L52I, I58V, L75R, F87A, H100R, S106R, F107L, A135S, A184V, N239H, S274T, L324I, V340M, I366V, K434E, E442K, V446I	0.001980	-100	N/A
22A3	13C9R1 F162I E434K K442E I446V	0.004053	-70	4
2C6 (*27*)	**9-10A** A78L, F87A, V184T, G315S, A330V	0.004257	-78	-15
9C7 (*27*)	**9-10A** C47R, A78L, F87G, I94K, A180V, V184T, G315S, A330V, Y345C	0.007258	-79	-5
B1 (*27*)	**9-10A** C47R, A78L, F87A, I94K, V184T, I263M, G315S, A330V	0.002246	-61	-14
B1SYN (*27*)	**9-10A** C47S, N70Y, A78L, F87A, I174N, I94K, V184T, I263M, G315S, A330V	0.002705	-76	-23
H2-4-D4	**9-10A TS** F87V, L75A, M177A, L181A, T268A, L437A	0.052439	57	-84
E12 A87V (*27*)	**9-10A** C47R, A78L, F87V, I94K, A111V, V141I, A180V, V184T, G315S, A330V	0.001990	-65	-52
GlcA4 T180A	**9-10A** C47R, F81W, A82S, F87A, I94K	0.004925	-78	12
H2-8-C7 (*26*)	**9-10A TS** F87V, L75A, L181A	0.000808	-100	N/A
CH-F8	**9-10A** L51A, C47A, F87V,	0.001126	-100	N/A

	I94K, L181A, C205F, S254R, I366V, L437A, E442K			
H2-4-H5 (*26*)	**9-10A TS** F87V, L75A, M177A, L181A	0.001229	-100	N/A
SA9	**9-10A** C47R, F81W, A82I, F87A, I94K, A180T, A197V	0.004170	-81	11
ManA10	**9-10A** C47R, F81S, A82V, F87A, I94K, A180T, A197V	0.006340	-82	14
Man1	**9-10A** C47R, F81L, A82T, F87A, I94K	0.003053	-73	21
MB2	**9-10A** C47R, F81W, A82I, F87A, I94K	0.003282	-77	10
HA62	**9-10A** C47R, F81A, A82L, F87A, I94K	0.003375	-81	-5
9-10A TS	V78A, P142S, T175I, A184V, S226R, H236Q, E252G, A290V, L353V, I366V, E442K	0.001920	-75	-54
9-10A TS F87A	**9-10A TS** F87A	0.001546	-60	5
25F7	**9-10A** C47R, A74F, A78S, F87A, I282K, C205F, S255R	0.001829	-81	43
24C4	**9-10A** C47R, A74I, A78L, F87A, I94K, C205F, S255R	0.000783	-100	N/A
5A1	**9-10A** M30T, C47R, A74F, A78S, I94K, C205F, S255R, Q310L, I366V, E442K	0.002471	-80	15
8B3	**9-10A** M30T, C47R, A74F, A78S, I94K, C205F, C255R, L310Q, Q323L, I366V, N381K, R398H, E441K	0.001315	-100	N/A

∗ Reported as the sum of the area of the cyclopropane peaks over the area of the internal standard. † Diastereomeric excess = ([cis]-[trans])/([cis]+[trans]). ‡ (*R*,*S*) – (*S*,*R*).

Table S6: Raw GC screening data for the chimeric P450s in the compilation plate

P450	chimeric P450s (heme domain block sequence)	absolute activity*	*de*†	*ee* (cis) ‡
CYP102A1 ($P450_{BM3}$) F87A (*28*)	11111111	0.001704	-28	56
CYP102A2 F88A (*28*)	22222222	N/A	N/A	N/A
CYP102A3 F88A (*28*)	33333333	N/A	N/A	N/A
5R1 (*29*)	32312231	0.008625	58	19
9R1 (*29*)	12112333	0.0042707	58	24
12R1 (*29*)	12112333	0.0701514	32	-49
C1D11R1 (*29*)	21113312	0.007138	51	9
C2B12R1 (*29*)	32313233	0.005914	38	-5
C2C12R1 (*29*)	21313111	0.006226	28	9
C2E6R1 (*29*)	11113311	0.008731	25	6
C2G9R1 (*29*)	22213132	0.007975	15	31
C3D10R1 (*29*)	22132231	0.004898	-16	-2
C3E4R1 (*29*)	21313311	0.007893	14	17
F3H12R1 (*29*)	21333233	0.005586	-56	-17
F6D8R1 (*29*)	22313233	0.008088	-76	-6
C3B5R1 (*29*)	23132233	0.014722	-81	4
X7R1 (*29*)	22312333	0.017305	-4	-34

* Reported as the sum of the area of the cyclopropane peaks over the area of the internal standard. † Diastereomeric excess = ([cis]-[trans])/([cis]+[trans]). ‡ (*R*,*S*) – (*S*,*R*).

Determining the cyclopropanation activity of the top 10 hits (highlighted in yellow on tables S5-6) under anaerobic conditions. Small-scale reactions (400 μL total volume) were conducted as described in section II and were analyzed by GC (cyclosil-B 30 m x 0.32 mm x 0.25 μm): oven temperature = 100 °C 5 min, 1 °C / min to 135 °C, 135 °C 10 min, 10 °C / min to 200 °C, 200 °C 5 min, *cis*-cyclopropanes (39.40 min and 40.20 min), *trans*-cyclopropanes (44.69 min and 45.00 min).

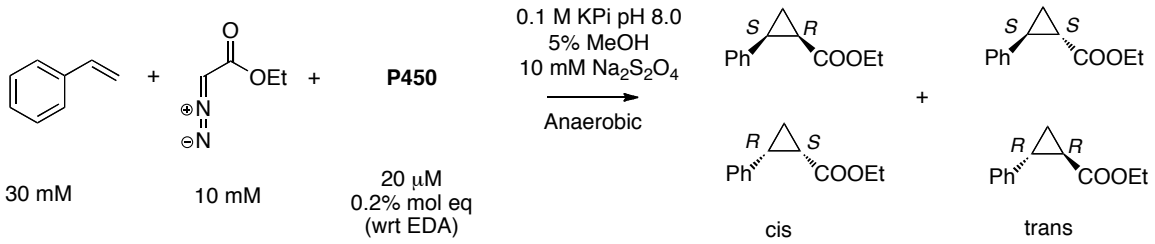


Table S7: Stereoselective $P450_{BM3}$-based cyclopropanation catalysts

P450	% yield*	TTN	cis : trans†	%ee cis‡	%ee trans§
WT	1	5	37 : 63	-10	-9
WTF87A	1.2	6	37 : 63	26	-6
H2A10	33.4	167	60 : 40	-95	-78
H2-4-D4	41.2	206	53 : 47	-79	-33
H2-5-F10	58.8	294	16 : 84	-41	-63
C2C12R1	1.6	8	36 : 64	45	1
C3E4R1	1.6	8	43 : 57	51	-7
X7R1	2.4	12	33 : 67	23	-4
12 R1	6.2	31	17 : 83	9	-2
C2E6 R1	4.6	23	27 : 73	25	-6
C2G9 R1	48	240	9 : 91	10	-2
7-11D	32	160	35 : 65	-22	-18

∗ based on EDA. † Diastereomeric ratios and enantiomeric excess were determined by GC analysis. ‡ (*R*,*S*) – (*S*,*R*). § (*R*,*R*) – (*S*,*S*).

V. Experimental Characterization of $P450_{BM3}$ Cyclopropanation Catalysts

V.I. Controls to confirm the enzymatic cyclopropanation activity of variant H2A10. Small-scale reactions (400 μL total volume) were set up and worked up as described in section II. For the carbon monoxide (CO) inhibition experiment, the reaction vial and the buffer/reductant vial were purged with CO after having been purged with argon. For the boiled P450 experiment, a 100 μM solution of variant H2A10 was heated at 60 °C for 10 min. For the hemin experiment, hemin (80 μL) was added from a 1 mM solution in 50% DMSO-H2O, such that its final concentration in the reaction was 200 μM. Complete System = 10 mM styrene, 20 mM EDA, 20 mM $Na_2S_2O_4$, 20 μM P450 (H2A10) under anaerobic conditions. The dried ethyl acetate extracts were analyzed by chiral phase GC, using 2-phenylethanol as an internal standard (injector temperature = 300 °C, oven temperature = 100 °C for 5 min, 1 °C / min ramp up to 135 °C, 135 °C for 10 min, 10 °C / min ramp up to 200 °C, 200 °C for 5 min). Elution time: *cis*-cyclopropanes (39.40 min and 40.20 min), *trans*-cyclopropanes (44.69 min and 45.00 min).

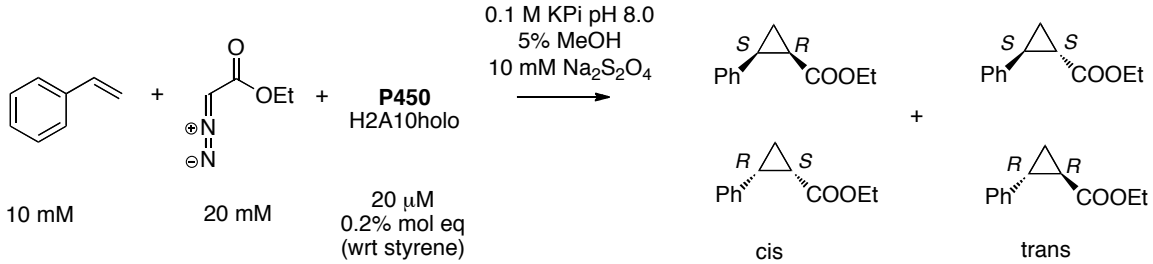


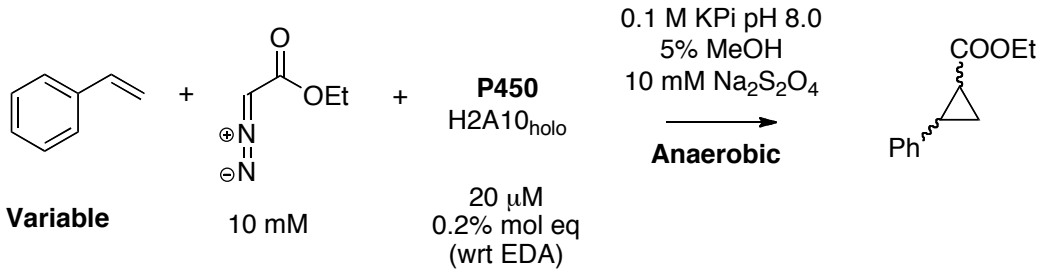
Table S8: Controls for P450 based cyclopropanation using variant H2A10

conditions	TTN	% inhibition	cis : trans*	% *ee* cis†	% *ee* trans‡
complete system (CS)	101	-	70 : 30	-95	-78
CS-$Na_2S_2O_4$+NADPH	45	-55	61 : 39	-87	-31
CS-$Na_2S_2O_4$+NADH	38	-62	53 : 47	-76	-19
CS-$Na_2S_2O_4$	0	-100	-	-	-
CS-P450	0	-100	-	-	-
CS+CO	0	-100	-	-	-
boiled P450	146	+45	16 : 84	2	-2
$H2A10_{heme}$	85	-16	67 : 33	-92	-67
CS-P450+hemin	16	-84	15 : 85	-1	-2
CS (aerobic)	43	-57	67 : 33	-94	-76

* Diastereomeric ratios and enantiomeric excess were determined by GC analysis. † (*R*,*S*) – (*S*,*R*). ‡ (*R*,*R*) – (*S*,*S*).

V.II. Optimizing cyclopropanation reaction conditions for variant H2A10. Small-scale reactions (400 µL final volume) were set up and worked up as described in section II. The dried ethyl acetate extracts were analyzed by chiral phase GC, using 2-phenylethanol as an internal standard (injector temperature = 300 °C, oven temperature = 100 °C for 5 min, 5 °C / min ramp up to 200 °C, 20 °C / min ramp up to 250 °C, 250 °C for 5 min). Elution time: *cis*-cyclopropanes (19.20 min and 19.33 min), *trans*-cyclopropanes (20.44 min). The reaction conditions that gave optimal yields of cyclopropanes (with respect to EDA) were: 30 mM styrene, 10 mM EDA and 20 µM P450 and were used in subsequent experiments.

V.II.I. Styrene concentration



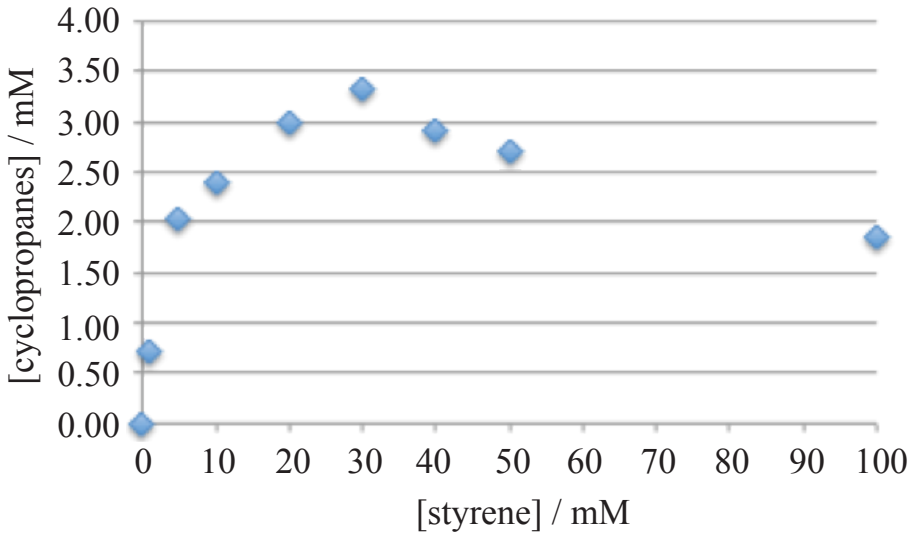
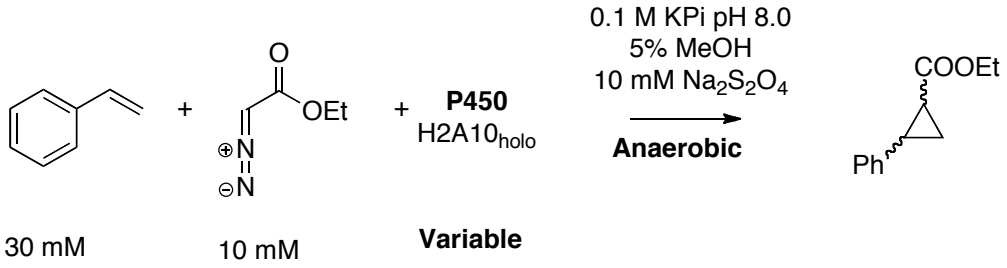


Figure S1: Effect of styrene concentration on cyclopropane yield.

V.II.II. P450 concentration



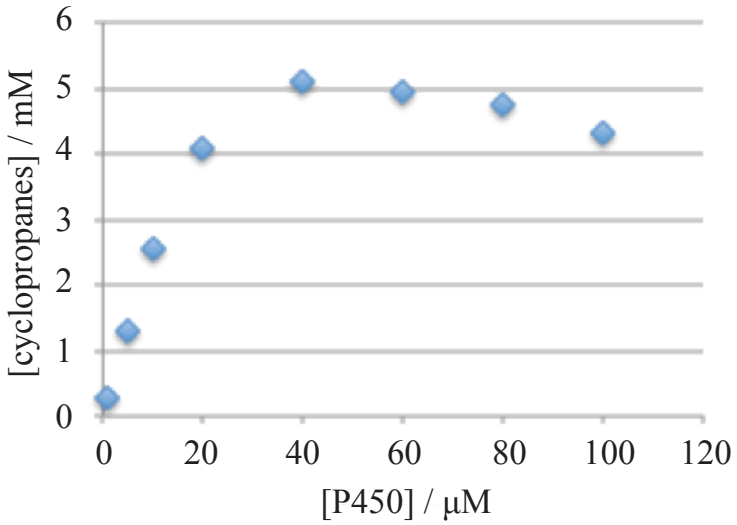


Figure S2: Effect of P450 (H2A10) concentration on cyclopropane yield

V.II.III. Dithionite concentration

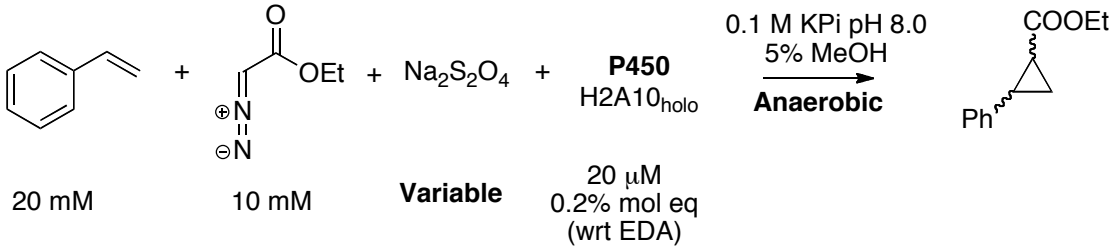


Table S9: Effect of concentration of $Na_2S_2O_4$ on cyclopropane yield

$[Na_2S_2O_4]$ / mM	[cyclopropanes] / mM	TTN
0	0	0
1	2.59	129
5	2.72	136
10	3.34	167
20	3.13	156
50	2.79	140
100	2.71	136

V.III. Mutational analysis of active site alanine substitutions in 9-10A TS F87V

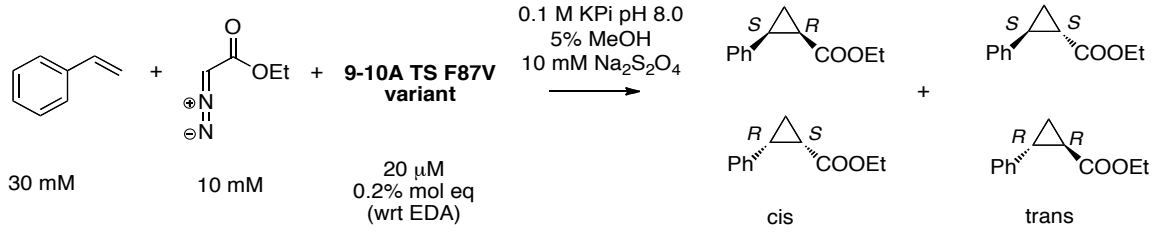


Table S10: Mutational analysis of alanine substitutions on 9-10A TS F87V

Mutations relative to 9-10A TS F87V

P450 (Holo)	L75A	M177A	L181A	I263A	T268A	L437A	TTN	cis : trans*	%ee cis†	%ee trans‡	T_{50} (°C)
9-10A TS F87V	No	No	No	No	No	No	7	35 : 65	-41	-8	59.5
9-10A TS F87V L75A	Yes	No	No	No	No	No	5	42 : 58	-59	-11	52.3
9-10ATS F87V L181A	No	No	Yes	No	No	No	5	41 : 59	-27	-7	53.3
9-10A TS F87V I263A	No	No	No	Yes	No	No	8	29 : 71	-31	-39	55.4
9-10A TS F87V T268A (BM3-CIS)	No	No	No	No	Yes	No	199	71 : 29	-94	-91	55.2
BM3-CIS I263A	No	No	No	Yes	Yes	No	190	19 : 81	-62	-91	54.0
BM3-CIS L181A	No	No	Yes	No	Yes	No	159	56 : 44	-92	-94	50.8
H2A10	Yes	No	Yes	No	Yes	No	167	60 : 40	-95	-78	48.9
BM3-CIS L181A I263A	No	No	Yes	Yes	Yes	No	203	14 : 86	-46	-95	50.9
BM3-CIS L181A L437A	No	No	Yes	No	Yes	Yes	180	27 : 73	-74	-98	48.4
BM3-CIS L181A I263A L437A	No	No	Yes	Yes	Yes	Yes	218	9 : 91	-55	-96	48.2
4H5	Yes	Yes	Yes	No	No	No	7	32 : 68	-9	0	49.4
BM3-CIS I263A L437A	No	No	No	Yes	Yes	Yes	267	16 : 84	-59	-89	50.4
H2-5-F10	Yes	No	No	Yes	Yes	Yes	294	16 : 84	-41	-63	47.5
H2-4-D4	Yes	Yes	Yes	No	Yes	Yes	206	53 : 47	-79	-33	46.4

* Diastereomeric ratios and enantiomeric excess were determined by GC analysis. † (*R*,*S*) – (*S*,*R*). ‡ (*R*,*R*) – (*S*,*S*).

V. IV. Sequential introduction of BM3-CIS active site mutations in wild-type $P450_{BM3}$

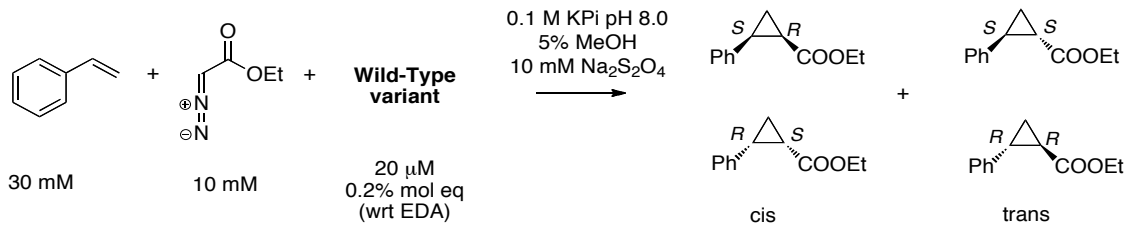


Table S11: Introducing BM3-CIS related active site mutations in wild-type $P450_{BM3}$

	Mutations relative to wild-type $P450_{BM3}$								
P450 (Holo)	**V78**	**F87**	**T268**	**I263**	**TTN**	**cis : trans***	**%ee cis†**	**%ee trans‡**	**T_{50} (°C)**
WT	-	-	-	-	5	37 : 63	-10	-9	56.0
WT-F87A	-	A	-	-	6	38 : 62	26	-6	53.0
WT-F87V	-	V	-	-	9	30 : 70	-33	-26	52.9
WT-T268A	-	-	A	-	323	1 : 99	-15	-96	53.6
WT-F87V/T268A	-	V	A	-	274	32 : 68	-77	-99	52.0
WT-V78A/F87V/T268A	A	V	A	-	190	32 : 68	-70	-20	50.8
WT-F87V/I263A/T268A	-	V	A	A	246	7 : 93	8	-94	50.0

* Diastereomeric ratios and enantiomeric excess were determined by GC analysis. † (*R*,*S*) – (*S*,*R*). ‡ (*R*,*R*) – (*S*,*S*).

VI. Active Site Saturation Mutagenesis of BM3-CIS$_{heme}$

Library construction. To simplify library construction and screening, only the BM3-CIS heme domain, which comprises residues 1-462 was used. This truncated enzymes lacks the P450 native reductase and exhibits similar activity and stereochemical control to the full length enzyme using $Na_2S_2O_4$ as a reductant, but not NADPH (data not shown). P450 site-directed mutagenesis and site-saturation libraries were assembled from PCR fragments generated from oligonucleotides containing the desired codon mutation or a degenerate NNK (or for reverse primers, the reverse complement MNN; where N = A,T,G,C, K = G,T and M = A,C) codon, which codes for all 20 amino acids and the TAG stop codon. PCR fragments were assembled using either standard overlap extension PCR or through restriction cloning using the Type IIS restriction enzyme, BsaI, depending on convenience.

Lysate screening under aerobic conditions. The compilation plate was expressed and lysed as described in section II (**enzyme library screening**). 150 μL lysate was transferred (Multimek 96-channel pipetting robot, Beckman Coulter, Fullerton, CA) to a 2 mL deep-well plate, with 50 μL of 120 mM $Na_2S_2O_4$ in 0.1 M KPi (pH = 8.0). 100 μL of a 90 mM styrene, 30 mM EDA mixed solution in 15% MeOH in 0.1 M KPi (pH = 8.0) was added to the plate to initiate the reaction. The plate was sealed and was left shaking (300 rpm) for four hours. The plastic seal was removed and 30 μL HCl (3 M) was added to quench the reaction followed by 20 μL of an internal standard solution (20 mM 2-phenylethanol in methanol). Acetonitrile (400 μL) was added before carefully vortexing the plate. The plate was centrifuged (1,700xg), the supernatant was filtered (1 μm glass, 96 well filter plate, Pall) and transferred (150 μL) to a 96-well microtiter plate (Agilent). Reactions were analyzed by reverse-phase HPLC (210 nm): 50% acetonitrile-water, 1.0 mL min^{-1}, *cis*-cyclopropanes (7.6 min), *trans*-cyclopropanes (9.7 min). Hits were selected based on enhancement of *cis*-selectivity over parent BM3-CIS.

Determining the cyclopropanation activity of hits from the site-saturation libraries under anaerobic conditions. Small-scale reactions (400 µL total volume) were conducted as described in section II and were analyzed by GC (cyclosil-B 30 m x 0.25 mm x 0.25 µm): oven temperature = 130 °C, 175 kPa, *cis*-cyclopropanes (39.40 min and 40.20 min), *trans*-cyclopropanes (44.69 min and 45.00 min).

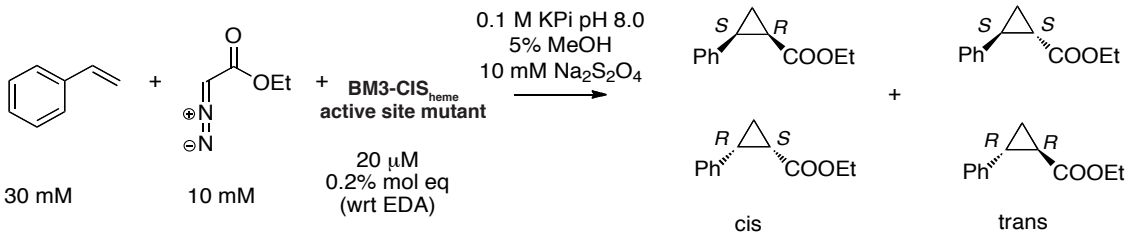


Table S12: Cyclopropanation activity of selected BM3-CIS$_{heme}$ active site variants

P450$_{heme}$	yield (%)*	TTN	cis : trans†	%ee cis‡	%ee trans§
BM3-CIS	57	286	71 : 29	-92	-88
BM3-CIS-L181G	47	234	59 : 41	-89	-90
BM3-CIS-A328G	37	186	83 : 17	52	-45
BM3-CIS-L437F	53	265	53 : 47	-82	-85
BM3-CIS-L437Q	30	148	53 : 47	-73	-87
BM3-CIS-L437G	58	290	54 : 46	-88	-91
BM3-CIS-L437A	39	194	38 : 62	-84	-11
BM3-CIS-T438A	54	273	91 : 9	-92	-75
BM3-CIS-T438G	15	78	73 : 27	-87	-59
BM3-CIS-T438S	59	293	92 : 8	-97	-66
BM3-CIS-T438Q	41	206	38 : 62	67	70
BM3-CIS-T438P	32	161	90 : 10	-91	-50

* based on EDA. † Diastereomeric ratios and enantiomeric excess were determined by GC analysis. ‡ (*R*,*S*) – (*S*,*R*). § (*R*,*R*) – (*S*,*S*).

VII. Kinetic Characterization of BM3-CIS

Determination of initial rates. Both styrene and EDA concentrations were varied in the presence of the P450s expressed as the heme-domain (0.5 or 1.0 μM BM3-CIS$_{heme}$). Reactions were set up in phosphate buffer (pH = 8.0) with $Na_2S_2O_4$ as the reductant at 298 K, and were worked-up as described in section II. Three time points were taken and used to determine the rate of product formation by GC (cyclosil-B 30 m x 0.32 mm x 0.25 μm): oven temperature = 100 °C 5 min, 5 °C / min to 200 °C, 20 °C / min to 250 °C, 250 °C for 5 min. Elution time: *cis*-cyclopropanes (19.20 min and 19.33 min), *trans*-cyclopropanes (20.44 min). Kinetic parameters were determined by fitting the data to the standard Michaelis-Menten model.

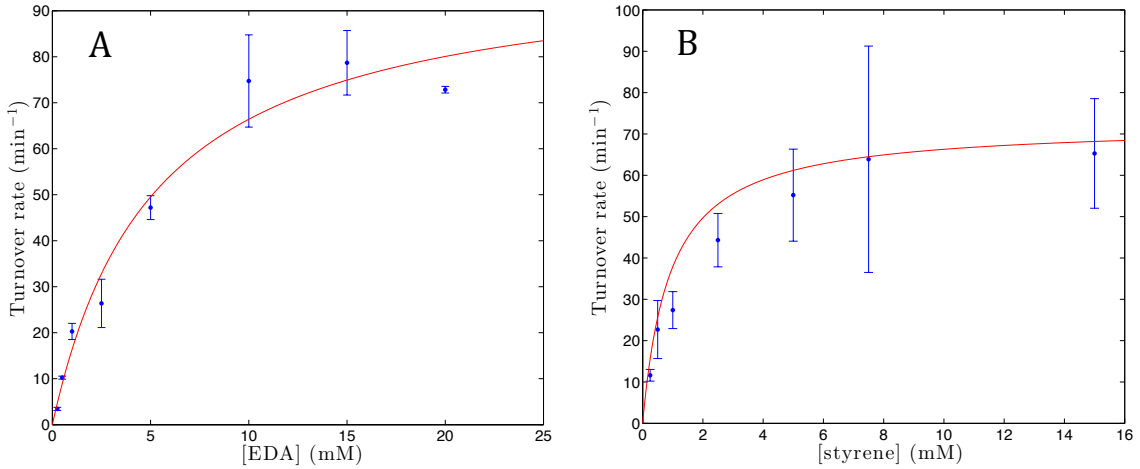


Figure S3: Initial velocities plot for BM3-CIS$_{heme}$. A) EDA concentration was varied at a saturating concentration of styrene (30 mM). B) Styrene concentration was varied at a fixed concentration of EDA (20 mM). Initial rates were computed as the slope of a zero-intercept linear fit of three different time points from independent reactions. Error bars correspond to 1-σ (68.3%) confidence intervals for the slope.

Table S13: Michaelis-Menten parameters for P450 cyclopropanation catalysts

catalyst	k_{cat} (min^{-1})	$K_{M\text{-}EDA}$ (mM)	$K_{M\text{-}styrene}$ (mM)	k_{cat} / $K_{M\text{-}EDA}$ (s^{-1} M^{-1})	k_{cat} / $K_{M\text{-}styrene}$ (s^{-1} M^{-1})	k_{cat} / ($K_{M\text{-}EDA}$ x $K_{M\text{-}styrene}$) (s^{-1} M^{-1} M^{-1})
BM3-CIS$_{heme}$	100 ± 24	5.2 ± 3.5	1.4 ± 0.5	320	1,100	2.1 x 10^5

Table S14: Kinetic parameters for wild-type cytochrome P450s acting on their native substrates and for an engineered variant of $P450_{BM3}$ (propane monooxygenase, PMO) acting on the non-native substrate propane

P450	**substrate**	k_{cat} **(min^{-1})**	K_M **(mM)**	k_{cat} / K_M **(s^{-1} M^{-1})**
CYP153A6 (*30*)	octane	75	0.32	3,900
$P450_{BM3}$ (*31*)	lauric acid	5140	0.29	3.0×10^5
PMO (*32*)	propane	450	0.17	4.4×10^4

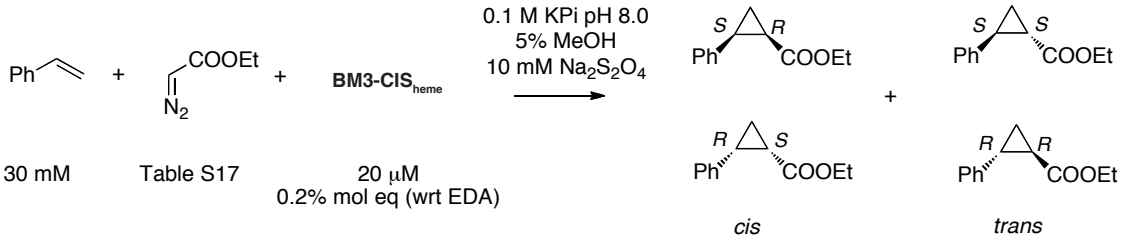


Table S15: Effect of EDA addition at t = 30 min on BM3-CIS-catalyzed cyclopropanations

conditions	TTN	cis : trans*	%ee cis †	%ee trans‡
10 mM EDA added at t = 0	273 ± 2.5	72 : 28	-92	-90
10 mM EDA added at t = 0 + 10 mM EDA at t = 30 min	425 ± 17	73 : 27	-93	-89

TTN values are reported as the mean of triplicates ± standard deviation. * Diastereomeric ratios and enantiomeric excess were determined by GC analysis. † (*R*,*S*) – (*S*,*R*). ‡ (*R*,*R*) – (*S*,*S*)

VIII. Substrate Scope of P450 Cyclopropanation Catalysts

Small-scale reactions. Selected P450 catalysts were surveyed at a small-scale (400 μL total volume) for each combination of reagents (olefins and diazo esters). The small-scale anaerobic bioconversions were conducted as described in section II and were analyzed by GC.

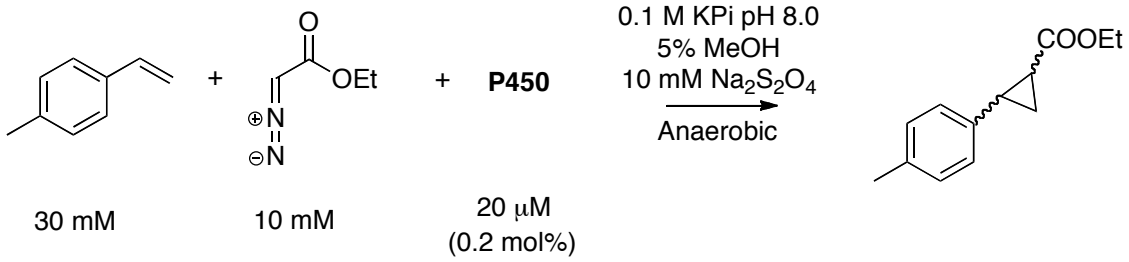


Table S16: Substrate scope of P450 cyclopropanation catalysts: *p*-methylstyrene + EDA

P450	% yield	TTN	cis : trans	%ee cis	%ee trans*
7-11D	21	104	54 : 46	0.3	N/A
H2-5-F10	44	222	11 : 89	14.9	N/A
C2G9 R1	18	92	10 : 90	8.9	N/A
H2A10	10	50	43 : 57	-84.3	N/A
BM3-CIS	46	228	78 : 22	-81.4	N/A
Hemin	7	37	6 : 94	-1.6	N/A

GC (cyclosil-B column 30 m x 0.32 mm, 0.25 μm film): oven temperature = 100 °C for 5 min, 5 °C / min to 200 °C, 20 °C / min to 250 °C, 250 °C for 5 min. Elution times: *cis*-cyclopropanes (21.03 and 21.18 min), *trans*-cyclopropanes (22.71 min). * *trans*-enantiomers did not resolve.

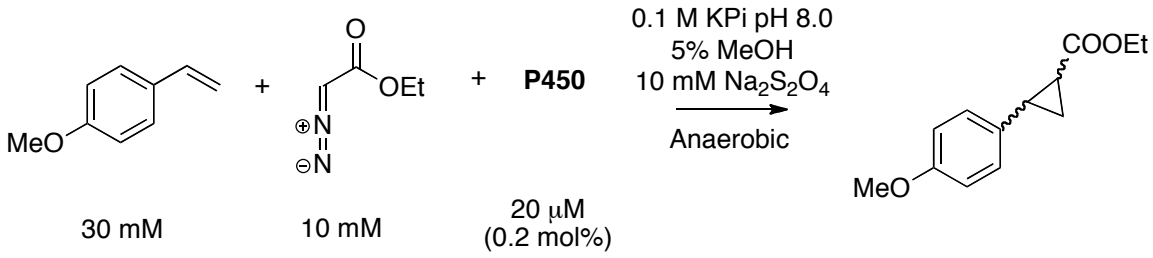


Table S17: Substrate scope of P450 cyclopropanation catalysts: *p*-vinylanisole + EDA

P450	% yield	TTN	cis : trans	%ee cis	%ee trans*
7-11D	59	297	70 : 30	-27	N/A
H2-5-F10	73	364	11 : 89	38	N/A
C2G9 R1	39	196	10 : 90	-1	N/A
H2A10	16	80	40 : 60	-75	N/A
BM3-CIS	43	214	48 : 52	-44	N/A
hemin	19	96	7 : 93	0	N/A

GC oven temperature = 110 °C for 8 min, 2 °C / min to 180 °C then 180 °C for 30 min, 175 kPa. Cyclosil-B column (30 m x 0.25 mm, 0.25 μm film). Elution times: *cis*-cyclopropanes (38.74 and 39.52 min), *trans*-cyclopropanes (43.07 min). * Baseline resolution could not be achieved for the *trans*-enantiomers.

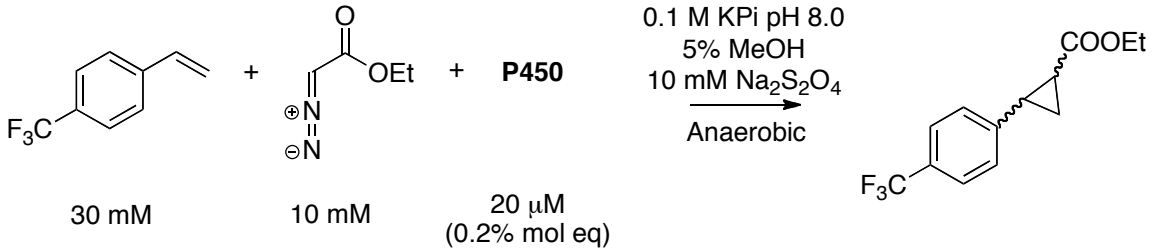


Table S18: Substrate scope of P450 cyclopropanation catalysts: *p*-(trifluoromethyl)styrene

P450	% yield*	TTN*	cis : trans	%ee cis	%ee trans
7-11D	24	120	76 : 24	31	59
H2-5-F10	40	198	26 : 74	72	-65
C2G9 R1	18	89	10 : 90	4	0
H2A10	9	47	26 : 74	-24	22
BM3-CIS	42	211	39 : 61	54	-93
hemin	2	9	11 : 89	1	1

* Assumed the same detector response factor as for ethyl 2-(4-methylphenyl)cyclopropane-1-carboxylate. GC (cyclosil-B column 30 m x 0.25 mm, 0.25 μm film): oven temperature = 110 °C for 8 min, 2 °C / min to 180 °C then 180 °C for 30 min, 175 kPa. Elution times: *cis*-cyclopropanes (27.26 and 28.11 min), *trans*-cyclopropanes (30.78 and 30.99 min).

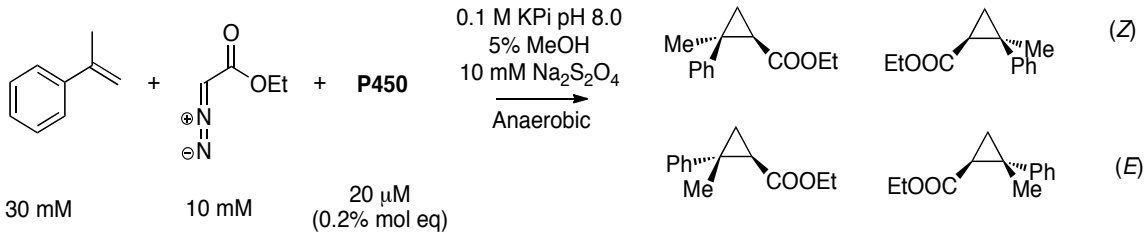


Table S19: Substrate scope of P450 cyclopropanation catalysts: α-methyl styrene

P450	% yield	TTN	Z : E	%ee (Z)	%ee (E) *
7-11D	31	157	41 : 49	42	N/A
H2-5-F10	66	329	21 : 79	-14	N/A
C2G9 R1	77	387	16 : 84	-4	N/A
H2A10	34	168	19 : 81	-31	N/A
BM3-CIS	26	127	16 : 84	-6	N/A
WT F87V T268A	62	312	7 : 93	3	N/A
hemin	15	77	24 : 76	0	N/A

GC oven temperature = 100 °C for 5 min, 1 °C / min up to 135 °C, 135 °C for 10 min, 10 °C / min up to 200 °C, 200 °C for 5 min. Cyclosil-B column (30 m x 0.32 mm, 0.25 μm film). Elution times: *Z*-cyclopropanes (34.96 and 35.33 min), *E*-cyclopropanes (39.34 and 39.61 min). * *trans*-enantiomers did not separate to baseline resolution.

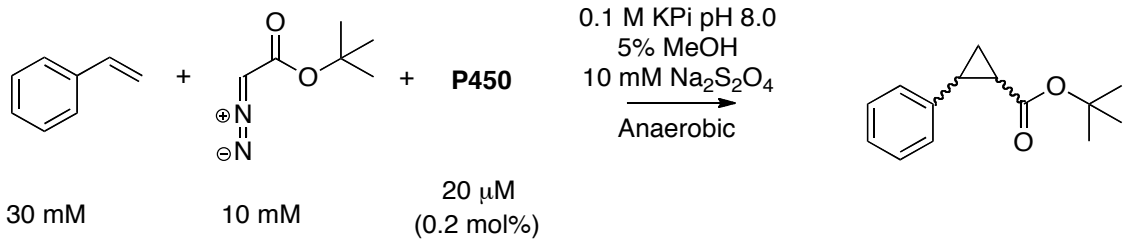


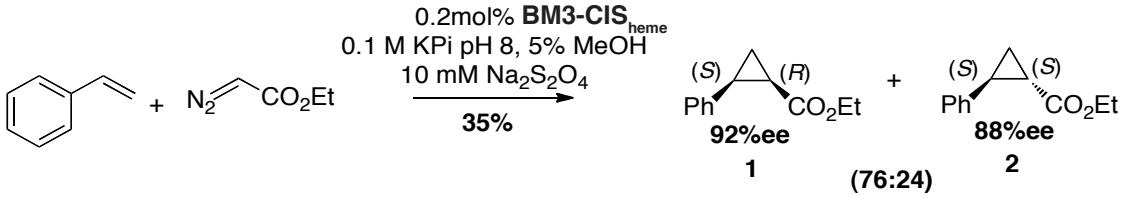
Table S20: Substrate scope of P450 cyclopropanation catalysts: *t*-butyl diazoacetate

P450	% yield*	TTN*	cis : trans
WT F87V T268A	1.4	7	4 : 96
7-11D	11	54	13 : 87
H2-5-F10	18	90	3 : 97
H2A10	24	120	3 : 97
BM3-CIS	0.3	2	3 : 97
hemin	20	100	4 : 96

* Assumed the same detector response factor as for ethyl 2-(4-methylphenyl)cyclopropane-1-carboxylate. GC (cyclosil-B column 30 m x 0.32 mm, 0.25 μm film): oven temperature = 100 °C for 5 min, 5 °C / min to 200 °C, 20 °C / min to 250 °C, 250 °C for 5 min. Elution times: *cis*-cyclopropanes (21.66 min), *trans*-cyclopropanes (23.31 min). *Cis*- and *trans*-enantiomers did not resolve.

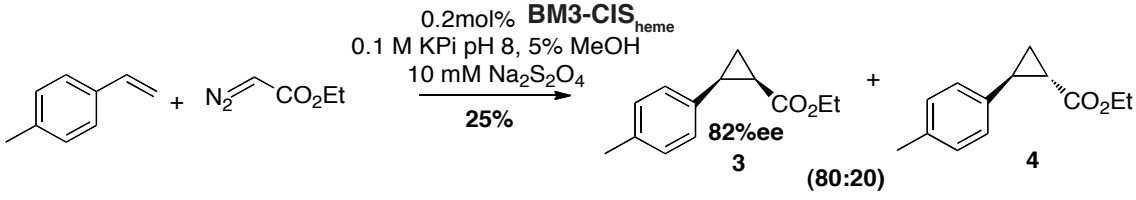
Preparative-scale bioconversions. These reactions were conducted anaerobically as described in section II.

Cyclopropanation of styrene with EDA



Prepared using 1.5 mmol styrene (3 equiv), 0.5 mmol EDA (1 equiv) and 1 µmol BM3-CIS_{heme} (0.002 equiv). The product was purified by SiO_2 chromatography (9:1 hexanes-diethyl ether) to give 25 mg of the *cis*-cyclopropane (**1**) and 8 mg of a mixture of cyclopropanes with *trans* (**2**) in 5 : 1 excess over *cis* (*33-35*). Diagnostic data for the *cis*-cyclopropane **1**: ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.28 (m, 4H), 7.21 (m, 1H), 3.89 (q, J = 7.1 Hz, 2H), 2.60 (m, 1H), 2.10 (m, 1H), 1.73 (m, 1H), 1.35 (m, 1H), 0.99 (t, J = 7.1 Hz, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 170.99, 136.56, 129.31, 127.88, 126.63, 60.18, 25.47, 21.80, 14.02, 11.12; $[\alpha]^{25}_D$ = - 7.056° (*c* 0.83, $CHCl_3$). Diagnostic data for the *trans*-cyclopropane **2**: ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.20 (m, 3H), 7.03 (m, 2H), 4.10 (q, J = 7.1 Hz, 2H), 2.45 (m, 1H), 1.83 (m, 1H), 1.53 (m, 1H), 1.23 (m, 1H), 1.21 (t, J = 7.1 Hz, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 173.43, 140.13, 128.46, 126.55, 126.16, 60.72, 26.18, 24.20, 17.09, 14.27; $[\alpha]^{25}_D$ = + 199.2° (*c* 0.50, $CHCl_3$). MS (EI^+) *m/z*: 190 (M^+), 162 ($PhCH(CH_2)CHCO2^+$), 145 ($PhCH(CH_2)CHCO^+$). The absolute configuration of compounds **1** and **2** was determined by comparison of the sign of their optical rotations with that reported (*19*). The enantiomeric excess was determined to be 92% for the *cis*-cyclopropane and 88% for the *trans*-cyclopropane by GC.

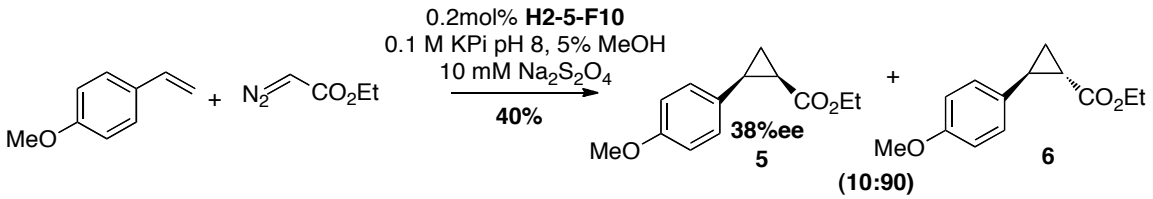
Cyclopropanation of *p*-methylstyrene with EDA



Prepared using 1.5 mmol styrene (3 equiv), 0.5 mmol EDA (1 equiv) and 1 µmol BM3-CIS_{heme} (0.002 equiv). The product was purified by SiO_2 chromatography (9:1 hexanes-diethyl ether) to give 10 mg of the *cis*-cyclopropane (**3**) and 16 mg of a mixture of cyclopropanes with *trans*(**4**) : *cis* / 2 : 1 (*35*). Diagnostic data for the *cis*-cyclopropane **3**: ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.17 (d, J = 8.0 Hz, 2H), 7.08 (d, J = 8.0 Hz, 2H), 3.91 (q, J = 7.1 Hz, 2H), 2.56 (m, 1H), 2.32 (s, 3H) 2.06 (m, 1H), 1.69 (m, 1H), 1.32 (m, 1H), 1.02 (t, J = 7.1 Hz, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 171.12, 136.12, 133.42, 129.14,

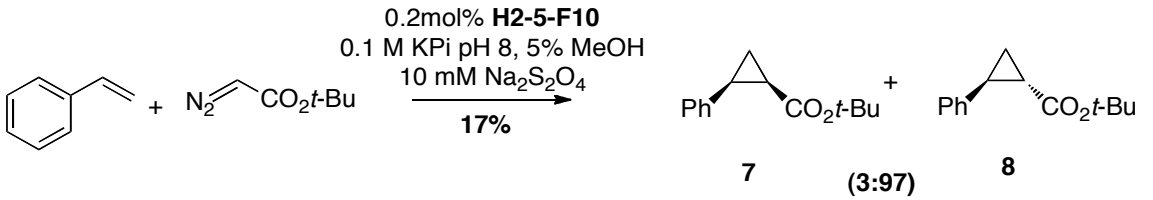
128.60, 60.17, 25.23, 21.68, 21.10, 14.08, 11.21. Diagnostic data for the *trans*-cyclopropane **4**: ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.09 (d, J = 8.0 Hz, 2H), 7.01 (d, J = 8.0 Hz, 2H), 4.19 (q, J = 7.1 Hz, 2H), 2.50 (m, 1H), 2.33 (s, 3H), 1.88 (m, 1H), 1.59 (m, 1H), 1.33 (m, 1H), 1.29 (t, J = 7.1 Hz, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 173.58, 137.04, 136.08, 129.12, 126.10, 60.66, 25.94, 24.06, 21.11, 16.96, 14.28. MS (EI^+) *m/z*: 204 (M^+), 175 ($[M\text{-}Et]^+$) 131 ($[M\text{-}COOEt]^+$). The enantiomeric excess was determined to be 82% for the *cis*-cyclopropane by GC. Baseline resolution of the *trans*-enantiomers could not be achieved.

Cyclopropanation of *p*-methoxystyrene with EDA



Prepared using 1.5 mmol styrene (3 equiv), 0.5 mmol EDA (1 equiv) and 1 μmol BM3-CIS_{heme} (0.002 equiv). The product was purified by SiO_2 chromatography (9:1 hexanes-diethyl ether) to give 16 mg of the *trans*-cyclopropane (**6**) and 3 mg of a mixture of cyclopropanes with *cis* : *trans* / 5 : 1 (*35*). Diagnostic data for the *trans*-cyclopropane **6**: 6.96 (m, 3H), 6.75 (m, 2H), 4.09 (q, J = 7.1 Hz, 2H), 3.72 (s, 3H), 2.41 (m, 1H), 1.75 (m, 1H), 1.48 (m, 1H), 1.21 (t, J = 7.1 Hz, 3H), 1.18 (m, 1H). MS (EI^+) *m/z*: 220 (M^+), 191 ($[M\text{-}Et]^+$), 175 ($[M\text{-}EtO]^+$), 147 ($[M\text{-}COOEt]^+$). The enantiomeric excess was determined to be 38% for the *cis*-cyclopropane by GC. The *trans*-enantiomers did not resolve to baseline resolution.

Cyclopropanation of styrene with *t*-butyl diazo acetate



Prepared using 0.75 mmol styrene (3 equiv), 0.24 mmol *t*-BuDA (1 equiv) and 0.5 μmol BM3-CIS_{heme} (0.002 equiv). The product was purified by SiO_2 chromatography (9:1 hexanes-diethyl ether) to give 9 mg of the *trans*-cyclopropane (**8**) (*33, 35*). Diagnostic data for the *trans*-cyclopropane **4**: ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.20 (m, 2H), 7.12 (m, 1H), 7.02 (m, 2H), 2.36 (m, 1H), 1.76 (m, 1H), 1.45 (m, 1H), 1.40 (s, 9H), 1.16 (m, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 172.58, 140.52, 128.42, 126.32, 126.07, 80.57, 28.17, 25.75, 25.31, 17.08. MS (EI^+) *m/z*: 218 (M^+), 145 ($[M\text{-}OtBu]^+$).

IX. Summary of mutations in $P450_{BM3}$ variants

Mutations in variant P450 cyclopropanation catalysts are reported with respect to wild-type $P450_{BM3}$.

7-11D (*24*): R47C, V78A, K94I, P142S, T175I, A184V, F205C, S226R, H236Q, E252G, R255S, A290V, L353V, A82F, A328V

9-10A TS (*26*): V78A, P142S, T175I, A184V, S226R, H236Q, E252G, A290V, L353V, I366V, E442K

H2A10: **9-10A TS +** F87V, L75A, L181A, T268A

H2-5-F10: **9-10A TS** + F87V, L75A, I263A, T268A, L437A

H2-4-D4: **9-10A TS** + F87V, L75A, M177A, L181A, T268A, L437A

BM3-CIS: **9-10A TS** + F87V, T268A

References and Notes

1. J. T. Groves, The bioinorganic chemistry of iron in oxygenases and supramolecular assemblies. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 3569 (2003). doi:10.1073/pnas.0830019100 Medline

2. R. Breslow, Biomimetic chemistry: Biology as an inspiration. *J. Biol. Chem.* **284**, 1337 (2009). doi:10.1074/jbc.X800011200 Medline

3. T. K. Hyster, L. Knörr, T. R. Ward, T. Rovis, Biotinylated Rh(III) complexes in engineered streptavidin for accelerated asymmetric C-H activation. *Science* **338**, 500 (2012). doi:10.1126/science.1226132 Medline

4. J. B. Siegel *et al*., Computational design of an enzyme catalyst for a stereoselective bimolecular Diels-Alder reaction. *Science* **329**, 309 (2010). doi:10.1126/science.1190239 Medline

5. H. M. L. Davies, J. R. Manning, Catalytic C-H functionalization by metal carbenoid and nitrenoid insertion. *Nature* **451**, 417 (2008). doi:10.1038/nature06485 Medline

6. H. Lebel, J.-F. Marcoux, C. Molinaro, A. B. Charette, Stereoselective cyclopropanation reactions. *Chem. Rev.* **103**, 977 (2003). doi:10.1021/cr010007e Medline

7. L. A. Wessjohann, W. Brandt, T. Thiemann, Biosynthesis and metabolism of cyclopropane rings in natural compounds. *Chem. Rev.* **103**, 1625 (2003). doi:10.1021/cr0100188 Medline

8. E. M. Isin, F. P. Guengerich, Complex reactions catalyzed by cytochrome P450 enzymes. *Biochim. Biophys. Acta, Gen. Subj.* **1770**, 314 (2007). doi:10.1016/j.bbagen.2006.07.003

9. J. R. Wolf, C. G. Hamaker, J.-P. Djukic, T. Kodadek, L. K. Woo, Shape and stereoselective cyclopropanation of alkenes catalyzed by iron porphyrins. *J. Am. Chem. Soc.* **117**, 9194 (1995). doi:10.1021/ja00141a011

10. B. Morandi, E. M. Carreira, Iron-catalyzed cyclopropanation in 6 M KOH with in situ generation of diazomethane. *Science* **335**, 1471 (2012). doi:10.1126/science.1218781 Medline

11. C. J. C. Whitehouse, S. G. Bell, L.-L. Wong, P450(BM3) (CYP102A1): Connecting the dots. *Chem. Soc. Rev.* **41**, 1218 (2012). doi:10.1039/c1cs15192d Medline

12. J. C. Lewis, F. H. Arnold, Catalysts on demand: Selective oxidations by laboratory-evolved cytochrome P450 BM3. *Chimia (Aarau)* **63**, 309 (2009). doi:10.2533/chimia.2009.309

13. Materials and methods are available as supplementary materials on *Science* Online.

14. A. Caballero, A. Prieto, M. M. Diaz-Requejo, P. J. Perez, Metal-catalyzed olefin cyclopropanation with ethyl diazoacetate: Control of the diastereoselectivity. *Eur. J. Inorg. Chem.* **2009**, 1137 (2009). doi:10.1002/ejic.200800944

15. I. Nicolas, P. Le Maux, G. Simonneaux, Asymmetric catalytic cyclopropanation reactions in water. *Coord. Chem. Rev.* **252**, 727 (2008). doi:10.1016/j.ccr.2007.09.003

16. U. T. Bornscheuer, R. J. Kazlauskas, Catalytic promiscuity in biocatalysis: Using old enzymes to form new bonds and follow new pathways. *Angew. Chem. Int. Ed.* **43**, 6032 (2004). doi:10.1002/anie.200460416

17. O. Khersonsky, C. Roodveldt, D. S. Tawfik, Enzyme promiscuity: Evolutionary and mechanistic aspects. *Curr. Opin. Chem. Biol.* **10**, 498 (2006). doi:10.1016/j.cbpa.2006.08.011 Medline

18. A. Penoni *et al*., Cyclopropanation of olefins with diazoalkanes, catalyzed by Co^{II}(porphyrin) complexes − a synthetic and mechanistic investigation and the molecular structure of Co^{III}(TPP)(CH_2CO_2Et) (TPP = dianion of meso-tetraphenylporphyrin). *Eur. J. Inorg. Chem.* **2003**, 1452 (2003). doi:10.1002/ejic.200390189

19. N. Watanabe, H. Matsuda, H. Kuribayashi, S.-i. Hashimoto, Dirhodium(II) tetrakis[3(S)-phthalimido-2-piperidinonate]: A novel dirhodium(II) carboxamidate catalyst for asymmetric cyclopropanation. *Heterocycles* **42**, 537 (1996). doi:10.3987/COM-95-S94

20. J. Sambrook, E. Frisch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, New York, 1989), vol. 2.

21. C. R. Otey, in *Methods in Molecular Biology: Directed Enzyme Evolution*, F. H. Arnold, G. Georgiou, Eds. (Humana Press, Totowa, NJ, 2003), vol. 230.

22. M. W. Peters, P. Meinhold, A. Glieder, F. H. Arnold, Regio- and enantioselective alkane hydroxylation with engineered cytochromes P450 BM-3. *J. Am. Chem. Soc.* **125**, 13442 (2003). doi:10.1021/ja0303790 Medline

23. A. Glieder, E. T. Farinas, F. H. Arnold, Laboratory evolution of a soluble, self-sufficient, highly active alkane hydroxylase. *Nat. Biotechnol.* **20**, 1135 (2002). doi:10.1038/nbt744 Medline

24. P. Meinhold, M. W. Peters, A. Hartwick, A. R. Hernandez, F. H. Arnold, Engineering cytochrome P450 BM3 for terminal alkane hydroxylation. *Adv. Synth. Catal.* **348**, 763 (2006). doi:10.1002/adsc.200505465

25. R. Fasan, M. M. Chen, N. C. Crook, F. H. Arnold, Engineered alkane-hydroxylating cytochrome P450BM3 exhibiting nativelike catalytic properties. *Angew. Chem. Int. Ed.* **46**, 8414 (2007). doi:10.1002/anie.200702616

26. J. C. Lewis *et al*., Combinatorial alanine substitution enables rapid optimization of cytochrome P450BM3 for selective hydroxylation of large substrates. *ChemBioChem* **11**, 2502 (2010). doi:10.1002/cbic.201000565 Medline

27. J. C. Lewis *et al*., Chemoenzymatic elaboration of monosaccharides using engineered cytochrome P450BM3 demethylases. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 16550 (2009). doi:10.1073/pnas.0908954106 Medline

28. C. R. Otey *et al*., Structure-guided recombination creates an artificial family of cytochromes P450. *PLoS Biol.* **4**, e112 (2006). doi:10.1371/journal.pbio.0040112

29. M. Landwehr, M. Carbone, C. R. Otey, Y. Li, F. H. Arnold, Diversification of catalytic function in a synthetic family of chimeric cytochrome p450s. *Chem. Biol.* **14**, 269 (2007). doi:10.1016/j.chembiol.2007.01.009 Medline

30. M. M. Chen, P. S. Coelho, F. H. Arnold, Utilizing terminal oxidants to achieve P450-catalyzed oxidation of methane. *Adv. Syn. Cat.* **354**, 964 (2012). doi:10.1002/adsc.201100833

31. M. A. Noble *et al*., Roles of key active-site residues in flavocytochrome P450 BM3. *Biochem. J.* **339**, 371 (1999). doi:10.1042/0264-6021:3390371 Medline

32. R. Fasan, Y. T. Meharenna, C. D. Snow, T. L. Poulos, F. H. Arnold, Evolutionary history of a specialized p450 propane monooxygenase. *J. Mol. Biol.* **383**, 1069 (2008). doi:10.1016/j.jmb.2008.06.060 Medline

33. C. J. Sanders, K. M. Gillespie, P. Scott, Catalyst structure and the enantioselective cyclopropanation of alkenes by copper complexes of biaryldiimines: The importance of ligand acceleration. *Tetrahedron Asymmetry* **12**, 1055 (2001). doi:10.1016/S0957-4166(01)00157-4

34. M. L. Rosenberg, A. Krivokapic, M. Tilset, Highly *cis* -selective cyclopropanations with ethyl diazoacetate using a novel Rh(I) catalyst with a chelating N-heterocyclic iminocarbene ligand. *Org. Lett.* **11**, 547 (2009). doi:10.1021/ol802591j Medline

35. Y. Chen, X. P. Zhang, Asymmetric cyclopropanation of styrenes catalyzed by metal complexes of D2-symmetrical chiral porphyrin: Superiority of cobalt over iron. *J. Org. Chem.* **72**, 5931 (2007). doi:10.1021/jo070997p Medline